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Sphingosine-1-phosphate (SPP), a bioactive sphingolipid metabolite, inhibits chemoinvasiveness of the aggressive, estrogen-independent MDA-MB-231 human breast cancer cell line. In contrast to its strong mitogenic effects on many other cell types, SPP only slightly stimulated proliferation of MDA-MB-231 cells. Treatment of MDA-MB-231 cells with SPP had no significant effect on the adhesiveness of cells to Matrigel, and high concentrations of SPP partially inhibited matrix metalloproteinase 2 activation induced by Con A. However, SPP at a concentration that strongly inhibited invasiveness also markedly reduced chemotactic motility. To investigate the molecular mechanisms by which SPP interferes with cell motility, we examined tyrosine phosphorylation of focal adhesion kinase (FAK) and paxillin, which are important for organization of focal adhesions and cell motility. SPP rapidly increased tyrosine phosphorylation of FAK and paxillin and of the paxillin-associated protein Crk. Overexpression of FAK and kinase-defective FAK in MDA-MB-231 cells resulted in a slight increase in motility without affecting the inhibitory effect of SPP, whereas overexpression of FAK with a mutation of the major autophosphorylation site (Y397F) abolished the inhibitory effect of SPP on cell motility. Our results suggest that autophosphorylation of FAK on Y397 may play an important role in SPP signaling leading to decreased cell motility.

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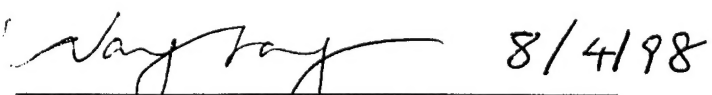

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INTRODUCTION

Metastasis is a multifactorial process by which tumor cells escape from the primary tumor, disseminate through blood and lymph vessels, evade host immune defense and home to specific target organs where they extravasate and re-colonize. Cell movement is considered a crucial step for the invasion of cancer cells [1, 2]. For cellular locomotion to occur, the cell must be able to protrude, form and break adhesion sites between the cell and the substratum, and move the cell mass. The assembly of focal adhesions, structures that link actin filaments and integrin-associated points of attachment with the extracellular matrix [3], and the concomitant recruitment of actin stress fibers and other cytoskeletal proteins is important for cell migration [4, 5]. Increased tyrosine phosphorylation of several proteins which are associated with focal adhesions has been implicated in cell migration and invasion [2, 5]. Among them, focal adhesion kinase (FAK), is a widely expressed and highly conserved non-receptor protein tyrosine kinase that has been implicated in organization of focal adhesions [6], formation of actin stress fibers [7, 8] and regulation of motility [9-11] and invasion [12, 13]. FAK, in turn, phosphorylates and/or associates with other cytoskeletal components, including Src, the Crk-associated substrate, p130Cas (Cas), paxillin and tensin [5, 14], that will either lead to activation of signaling pathways or establish contacts with talin, vinculin, a-actinin, and the actin polymers, essential components of stress fibers regulation.

Recent studies indicate that the sphingolipid metabolites, ceramide, sphingosine (Sph), and sphingosine-1-phosphate (SPP) can enhance tyrosine phosphorylation of FAK leading to stress fiber formation and focal contact assembly in Swiss 3T3 cells [7, 15]. Moreover, in human arterial smooth muscle cells, SPP generated in response to PDGF, interfered with the dynamics of PDGF-stimulated actin filament disassembly and assembly resulting in a marked inhibition of cell spreading, extension of the leading lamellae, and of chemotaxis toward PDGF [16]. These results suggest that endogenous SPP may play an important role in regulating cell migration and chemotactic signaling. In contrast, other studies suggest that SPP acts through a cell surface receptor to inhibit motility [17] and induce cell rounding [18]. At very low concentrations (10 nM), SPP, but not Sph, effectively inhibited chemotactic motility of several tumor cell lines, including mouse and human melanoma, and human osteosarcoma cells, whereas the motility of endothelial cells were not affected [17, 19]. However, the mechanism by which SPP inhibits motility is still not understood. In this study, we examined the effects

of SPP on invasiveness and motility of the highly invasive MDA-MB-231, human breast cancer (HBC) cell line, and also examined the involvement of FAK. Our experiments suggest that autophosphorylation of FAK on Y397 may play an important role in SPP signaling leading to a decrease in cell motility.

MATERIALS AND METHODS

Materials - The human breast cancer cell lines MDA-MB-231 and MCF-7 were obtained from the Cell Culture Core Resource, Lombardi Cancer Center (Washington, D.C.). Media and supplements were from Biofluids Inc. (Bedford, MA). Collagen type IV was purchased from Collagen Corp. (Palo Alto, CA). Polycarbonate filters were from Poretics (Livermore, CA). methyl^{3}H]Thymidine (55 Ci/mmol) was purchased from Amersham (Arlington Heights, IL). Sphingosine and SPP were obtained from Biomol Research Laboratory Inc. (Plymouth Meeting, PA). Sphingosine, N-hexanoyl sphingosine (C6-Cer) and N-acetyl sphingosine (C2-Cer) were from Matreya (Pleasant Gap, PA). Gelatin was from NOVEX (Encinitas, CA). Anti-FAK mAb was from Upstate Biotechnology Inc. (Lake Placid, NY). Anti-phosphotyrosine (PY20), anti-paxillin, anti-Crk and anti-β1integrin mAbs were obtained from Transduction Laboratories (Lexington, KY). Protein A/G-agarose was from Santa Cruz Biotechnology (Santa Cruz, CA). Bovine serum albumin, L-ascorbic acid and Coomassie Blue G-250 were from Sigma Chemical Co. (St. Louis, MO). Anti-KT3 antibody was from Babco (Richmond, CA).

Cell culture - Cells were maintained in Richter's Improved Minimal Essential Medium (IMEM) containing phenol red, supplemented with 5% fetal bovine serum (FBS). Unless indicated, cells were seeded at 1.8 x 10^4 cells/cm^2. 24 h prior to experiments, cells were changed to serum-free IMEM supplemented with 1% (v/v) HEPES, 1% (v/v) non essential amino acids, 1% (v/v) trace elements, 0.4% (v/v) ITS (Insulin, transferrin, selenium), 0.2% (w/v) fibronectin and 1% (v/v) vitamins, and treated with different reagents as indicated.

Chemoinvasion Assay - Boyden chamber chemoinvasion assays were carried out essentially as described previously using two kinds of chambers (growth area = 18 mm^2 and 50 mm^2)[20]. Briefly, polycarbonate filters (13 mm diameter, 12 μm pore size) were coated with Matrigel (25 μg), a murine tumor extract rich in basement membrane

components (particularly laminin, collagen type IV and heparan sulfate proteoglycan) for 1 h at 37°C, rinsed once with PBS, and then placed into the lower chamber. Fibroblast conditioned medium, obtained by incubating confluent NIH 3T3 cells for 24 h with IMEM supplemented with L-ascorbic acid (50 μg/ml), or fetal bovine serum (10%), were placed in the lower chamber as chemoattractants. Cells were harvested by trypsinization, washed twice with IMEM containing 0.1% bovine serum albumin (IMEM/BSA), and added to the upper chamber at $0.75x10^5$ (for small chamber) or $3x10^5$ cells per well (for large chamber). The sphingolipid metabolites were added to the lower chamber and the chambers incubated in a humidified incubator at 37°C in 5% CO_2/95% air for 6 h. The cells which traversed the Matrigel and spread on the lower surface of the filter were fixed in methanol for 8 minutes and stained with crystal violet. Nonmigratory cells on the upper membrane surface were removed with a cotton swab. The number of migratory cells per membrane was enumerated using light microscopy at 10X magnification. Each data point is the average number of cells in four random fields, each counted twice. Each determination represents the average of three individual wells, and error bars represent SD. In some experiments, cells were pretreated with the indicated sphingolipid metabolites for 24 h before the chemoinvasion assay.

[^{3}H]Thymidine incorporation - Cells were cultured in serum-free IMEM in the presence of different mitogens for 18 h and then pulsed with [^{3}H]thymidine (1 μCi/ml) for 6 h. The incorporation of radioactivity into trichloroacetic acid-insoluble material was then measured [21]. Values are the means of triplicate determinations and standard deviations were routinely less than 10% of the mean.

Flow cytometric analysis - Cells were treated without or with SPP for 24 h and trypsinized to obtain single cell suspensions. After centrifugation, cells (1×10^6) were resuspended in 100 μl citrate buffer (250 mM sucrose, 40 mM trisodium citrate and 0.05% (v/v) DMSO, pH 7.6). Cell nuclei were stained with propidium iodide and analysis of cell cycle distribution was performed with a FACStar^{plus} flow cytofluorometer (Becton Dickinson, San Jose, CA).

Adhesion assay - Matrigel (2.5 μg) was incubated in each well of a 96-well culture plate for 60 minutes at 37°C. The plates were then incubated with 3% BSA in PBS for 30 min to block non-specific binding sites followed by three washes with PBS. After treatment with the indicated amount of SPP for 6 or 24 h, cells were harvested by scraping in 10

mM EDTA in PBS, washed twice with IMEM/BSA, resuspended at 5 x 10^5 cells/ml in IMEM/BSA containing the indicated amounts of SPP, and incubated for 1 hour at 37°C. Cell suspensions (100 µl) were then added to each well and incubated at 37°C for 1 hour. Non-adherent cells were removed and the attached cells fixed with 70% ethanol for 20 minutes and stained with crystal violet (5 mg/ml in 20% methanol) for 10 minutes. Wells were gently rinsed three times with water and allowed to dry. Incorporated dye was dissolved in 100 µl/well of 0.1M sodium citrate in 50% ethanol (pH 4.2) and the absorbance measured at 540 nm.

Zymography - Cells in 24-cluster plates (5 x 10^4 cells/cm^2) were washed with IMEM and changed to serum-free conditioned medium (serum-free medium conditioned by MMP2-transfected MCF-7 cells as the source of latent MMP-2) as previously described [22]. Cells were treated with Con A in the absence or presence of SPP. The conditioned medium was collected after 24 h and MMP-2 activation analyzed by zymography on 10% SDS-PAGE gels copolymerized with 2 mg/ml gelatin (NOVEX, Encinitas, Calif.). The activity of the latent 72 kD species of MMP-2 can be visualized after exposure of the active site by SDS while MMP-2 activation is indicated zymographically by the size reduction to the intermediate and/or mature enzyme forms of 62 and 59 kD, respectively. Following electrophoresis, gels were washed with 2.5% Triton X-100 for 1 h at room temperature to remove SDS. Zymograms were developed by incubation overnight in collagenase buffer (0.2 M NaCl, 5 mM $CaCl_2$, 1% Triton X-100, and 0.02% NaN_3 in 50 mM Tris-HCl, pH 7.4). Zymograms were stained with 1% (w/v) Coomassie Blue G-250 dissolved in 30% methanol containing 10% glacial acetic acid. After destaining, gelatinolytic activities were visualized as clear bands against a dark background of stained gelatin.

Chemotaxis Assay - Chemotaxis was measured as described for the chemoinvasion assay with the exception that filter surfaces were coated with 5 µg of collagen IV instead of Matrigel [20]. Collagen IV coatings promote uniform attachment to and migration across the filter, without formation of a barrier. Since the cells can migrate without degrading the collagen, this assay measures chemotaxis rather than chemoinvasion.

Immunoprecipitation - Cells were treated with the indicated sphingolipids, washed twice with PBS and lysed at 4°C in 500 µl of lysis buffer consisting of 50 mM HEPES, pH 7.4/1% Triton X-100/150 mM NaCl/1 mM EDTA/1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride/50 mM NaF/4 mM sodium pyrophosphate/2 mM Na_3VO_4/

leupeptin and aprotinin (2.5 μg/ml each). After centrifugation at 14,500 x g, 350-500 μg aliquots of supernatant proteins were incubated with 3-4 μg monoclonal antibodies (mAbs) directed against FAK, paxillin, or Crk for 4 h at 4°C, followed by the addition of 20 μl of protein A/G-agarose. After incubating overnight at 4°C, the immune complexes bound to agarose were recovered by centrifugation, washed twice with lysis buffer, extracted for 10 minutes at 95°C in 2 X SDS-PAGE sample (200 mM Tris, 2 mM EDTA, 6% SDS, 4% 2-mercaptoethanol, 10% glycerol, pH 6.8), and then analyzed by SDS-PAGE.

Western Blotting - After SDS-PAGE, proteins were transferred to PVDF membranes, blocked with 3% BSA in PBS, and incubated with anti-Tyr(P) mAb (PY20H, 1:2500) or mAb directed against FAK, paxillin, or Crk as indicated. Immunoreactive bands were detected by ECL using horseradish peroxidase conjugated anti-mouse IgG. In some cases, the immunoblots were stripped and reprobed with antibodies according to the manufacturer's recommendations (ECL, Amersham Corp., Arlington Heights, IL).

Transfection - The cDNAs encoding FAK and its mutants contain an epitope tag derived from the final 11 COOH-terminal residues of SV40 large T antigen (KPPTPPPEPET). KD is a kinase-defective FAK mutant with a lysine 454 to arginine mutation in the ATP binding site of the kinase domain. F397 contains a mutation at the autophosphorylation site (Tyr-397 to Phe mutation). Epitope-tagged FAK and its mutants were cloned into vector pCDM8 to generate the expression plasmids, pCDM8-FAK, pCDM8-KD, and pCDM8-F397, as decribed previously [10] and were kindly provided by Dr. J.L. Guan (College of Veterinary Medicine, Cornell University, Ithaca, New York). MDA-MB-231 cells were cotransfected with 20 μg of the expression plasmids or vector alone together with 2 μg of pEGFP-C3 using the calcium phosphate precipitation method [23]. pEGFP-C3 encodes a green flueorent protein and contains a neomycin-resistance cassette. After 48 h, transfected cells expressing high levels of GFP were selected and isolated by FACS analysis. Neomycin-resistant cells were selected in growth media containing 0.9 mg/ml G418 (GIBCO BRL) for 20 days and maintained in the same media. Transfected cells were then screened for exogenous FAK expression by Western blotting using the mAb KT3.

RESULTS

SPP inhibits chemoinvasiveness of MDA-MB-231 cells

To examine whether sphingolipid metabolites affect invasion, MDA-MB-231 cells were pretreated with SPP, sphingosine, cell permeable ceramide analogs (C2-Cer and C6-Cer), or sphingomyelinase (which cleaves plasma membrane sphingomyelin to form ceramides) for 24 h, and then chemoinvasion towards fibroblast conditioned medium was measured. As can be seen in Fig. 1A, SPP, but not sphingomyelinase treatment or ceramide analogs inhibits invasion. A significant inhibitory effect was found at 5 μM SPP and a maximal effect was observed at 10-20 μM. Sphingosine, at 20 μM, has a smaller inhibitory effect. Similarly, SPP inhibits chemoinvasion towards fetal bovine serum (FBS). No cytotoxic effects were detected for any of these sphingolipids up to concentrations of 5 μM, but ceramide analogs were cytotoxic at concentrations ≥ 10 μM. In contrast, sphingosine and SPP were not cytotoxic up to 20 and 50 μM, respectively. Similar results were obtained when cells were treated with sphingosine or SPP for only 6 h during the chemoinvasion towards fibroblast conditioned medium and FBS (Fig. 1B). However, the inhibitory effects required higher concentrations when incubated with the cells for 6 h rather than 24 h.

Effects of sphingosine-1-phosphate on proliferation of MDA-MB-231 cells

Although SPP is a potent mitogen for many different cell types [24], it only induced a small increase in DNA synthesis of MDA-MB-231 (Fig. 2 A) and MCF-7 cells (data not shown) as measured by [^{3}H]thymidine incorporation. Consistent with these results, flow cytometric analysis of cell cycle distribution revealed that optimally mitogenic concentrations of SPP (10 μM) induced a slight increase in the proportion of MDA-MB-231 cells in S phase, with a concomitant decrease in the proportion of cells in Go-G1 (Fig. 2 B). It should be pointed out that despite the increase in S phase, no changes could be detected in cell numbers. In contrast, similar concentrations of SPP induced a significant increase in cell numbers of quiescent Swiss 3T3 fibroblasts [21].

SPP inhibits MMP-2 activation and has no effect on adhesion of MDA-MB-231 cells to Matrigel

There are three major steps of invasion: adhesion, extracellular matrix digestion, and cell movement. To determine whether the effect of SPP on invasion was due to altered adhesion, cells were pretreated with SPP or anti-integrin β1 antibody (20 μg/ml)

for 24 h and adherence to Matrigel was determined (Fig. 3 A). Treatment of MDA-MB-231 cells with SPP at concentrations that strongly inhibited invasiveness (5-10 μM) had no significant effects on the adhesiveness of cells to Matrigel. As expected, pretreatment of cells with integrin β1 antibody, strongly inhibited adhesion to Matrigel (Fig. 3A), while treatment of cells with a unrelated antibody had no effect.

To penetrate the extracellular matrix, metastatic cells disrupt local segments of the basement membrane with proteinases, such as MMP-2 [25]. In breast adenocarcinomas, MMP-2 is secreted by the reactive stroma and appears to be sequestered by the carcinoma cells [26]. Certain breast carcinoma cells, including MDA-MB-231, are able to activate MMP-2 when treated with Con A [22, 27]. MMP-2 is secreted as a proenzyme (72 kD) whose proteolytic activity is triggered by truncation and conformational rearrangement at the cell surface [28]. Due to the importance of activation of MMP-2 in matrix degradation in breast cancer [29], the effect of SPP on MMP-2 activation by MDA-MB-231 cells was examined. In agreement with previous studies [22, 27], Con A induced activation of MMP-2 (Fig. 3B, lane 2). Whereas SPP alone did not significantly affect MMP-2 activation (lane 4), it inhibited Con A-induced activation of MMP-2 only at high concentrations (lane 3). Thus, MMP-2 activation does not appear to be a major target of SPP in inhibition of chemoinvasion by these cells.

SPP inhibits chemotactic motility of MDA-MB-231 and MCF-7 cells

One of the critical steps for the invasion of cancer cells is cell moverment [1, 2]. Cell motility can be random (chemokinesis), directed towards concentration gradients of various attractants including growth factors (chemotaxis), or towards ECM-bound components (haptotaxis) [30]. Previously, low concentrations of SPP have been shown to affect motility of various types of cells [17, 19]. Thus, we examined the effects of SPP on chemoattractant-induced cell motility of MDA-MB-231 (Fig. 4A) and MCF-7 cells (Fig. 4B). As previously reported, MDA-MB-231 cells are much more motile than the MCF-7 cells (cell number per field was 1720 ± 80 for control MDA-MB-231 and 520 ± 75 for MCF-7 cells). SPP markedly inhibited chemotaxis at 5-10 μM in both cell lines. Similar to the results of the chemoinvasion assays, SPP was more potent than sphingosine at inhibiting motility of MDA-MB-231 cells (Fig. 4A). Cells treated with SPP for 6 h in the chamber without pretreatment also showed decreased motility compared with untreated cells (Fig. 4C). However, in this case, maximal inhibitory effects required higher concentrations of SPP.

SPP increases tyrosine phosphorylation of focal adhesion kinase

Little is yet known of the molecular mechanisms by which SPP regulates cell motility. Tyrosine phosphorylation and activation of FAK have been implicated in the regulation of cell motility [31]. Moreover, we have recently shown that SPP stimulates tyrosine phosphorylation of FAK in quiescent Swiss 3T3 fibroblasts [15], whereas no effect on FAK phosphorylation could be detected in human arterial smooth muscle cells whose chemotactic mobility was markedly inhibited by SPP [16]. To examine the effects of SPP on FAK phosphorylation in MDA-MB-231 cells, lysates were immunoprecipitated with anti-FAK mAb and analyzed by SDS-PAGE followed by immunoblotting with anti-phosphotyrosine antibody. SPP (10 μM) induced rapid tyrosine phosphorylation of FAK, reaching maximal levels within 5 min and declining thereafter (Fig. 5A). Treatment of cells with 10 μM sphingosine also induced tyrosine phosphorylation of FAK (Fig. 5B), whereas, the cell permeable ceramide analog C6-Cer had almost no effect (Fig. 5C).

SPP increased tyrosine phosphorylation of paxillin and Crk

In many cell types, FAK binds and phosphorylates other cytoskeletal components, particularly paxillin and tensin [5], that subsequently will establish contacts with other essential components of the cytoskeleton. SPP (10 μM) induced a time-dependent increase of paxillin phosphorylation with maximal effect at 5 min (Fig. 6A), declining after 15 min, and returning to basal levels after 60 min. Sphingosine (20 μM) also induced phosphorylation of paxillin within 5 min of treatment (Fig. 6B).

It has been suggested that phosphorylated paxillin may serve as a molecular adaptor, responsible for the recruitment of structural and signaling molecules to focal adhesions. The adoptor protein Crk has been shown to bind to tyrosine phosphorylated paxillin through its Src-homology 2 (SH2) domain [32] which may trigger downstream activation of MAP kinase. Similar to previous reports in other cell types [33, 34], SPP markely increased tyrosine phosphorylation of Crk. Within 5 min of treatment with SPP, there was a significant increase in tyrosine phosphorylation of the slower migrating Crk band without affecting that of the faster migrating protein (Fig. 6C). Sphingosine (20 μM) induced Crk phosphorylation to a less extent than SPP (10 μM). The two closely migrating proteins correspond to Crk as determined with Crk mAb.

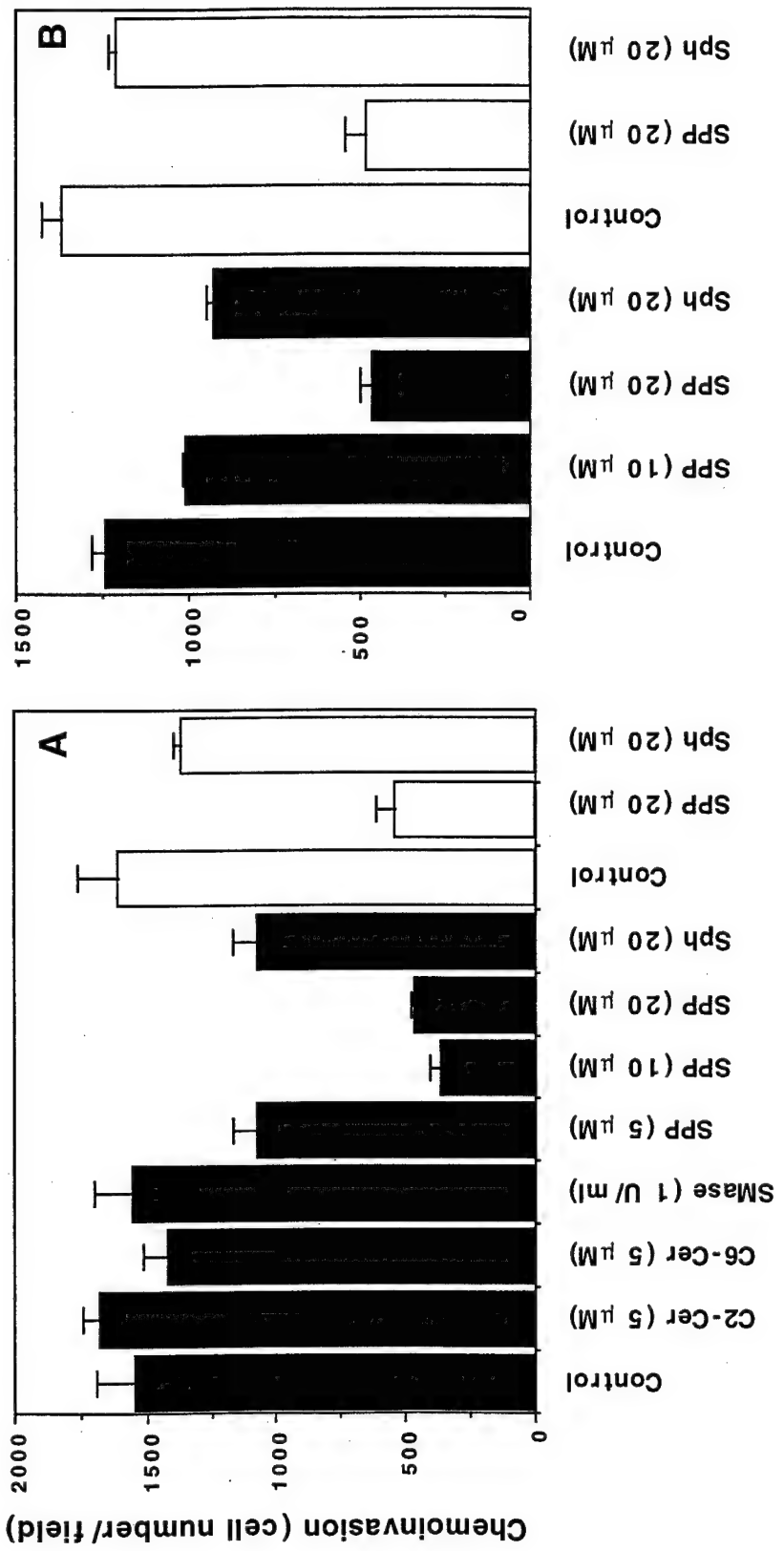
The major autophosphorylation site of FAK, Y397, is important for inhibition of motility of MDA-MB-231 cells by SPP

Recent studies demonstrate the importance of the major autophosphorylation site Y397 of FAK for CHO cell migration on fibronectin [10]. Overexpression of FAK or a kinase-defective FAK mutant in CHO cells resulted in increased migration wheras CHO cells expressing the FAK point mutant F397, which did not bind Src, demonstrated a basal level of cell migration. To examine the role of FAK in MDA-MB-231 cells we utilized these FAK constructs. After transfection and FACS isolation, cells stably expressing epitope-tagged WT, kinase-defective, or F397 FAK were screened by immunostaining with anti-KT3 antibody. A protein (125 kD) which was recognized by the KT3 antibody was detected in all three FAK transfected cell lines (lane 2-4) but not in control cells transfected with vector alone (Fig. 7A, upper panel). Total FAK expression was also increased in these three cell lines as detected by western blotting with anti-FAK antibody (Fig. 7A, lower panel).

To determine the effects of FAK phosphorylation on cell migration, chemotactic motility of these clones was analyzed by Boyden chamber assays. In agreement with previous studies [10], the WT and KD cell lines exhibited slightly increased levels of migration compared to vector-transfected cells, whereas expression of F397 resulted in slightly decreased motility. These results suggest that, similar to other cell types, autophosphorylation of Y397 of FAK is necessary for FAK-dependent motility of MDA-MB-232 cells.

Because SPP increased tyrosine phosphorylation of FAK and its associated substrates, it was of interest to examine the involvement of FAK in SPP signaling leading to decreased cell motility. SPP markedly inhibited motility of cells overexpressing WT and KD FAK but not in cells expressing autophosphorylation site mutated FAK. Although F397 FAK expressing cells have lower motility than WT cells, they still have a significant degree of motility. Moreover, the effect of SPP on WT cells was time-dependent (Fig. 7B), inhibition of motility was proportional to the length of time that the cells were exposed to SPP. However, SPP did not have any effect on motility of F397 cells at the time period examined.

Fig 1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1000
500
0
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C6-Cer (5 μM)
SMase (1 U/ ml)
SPP (5 μM)
SPP (10 μM)
SPP (20 μM)
Sph (20 μM)
Control
SPP (20 μM)
Sph (20 μM)
1500
1000
500
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SPP (10 μM)
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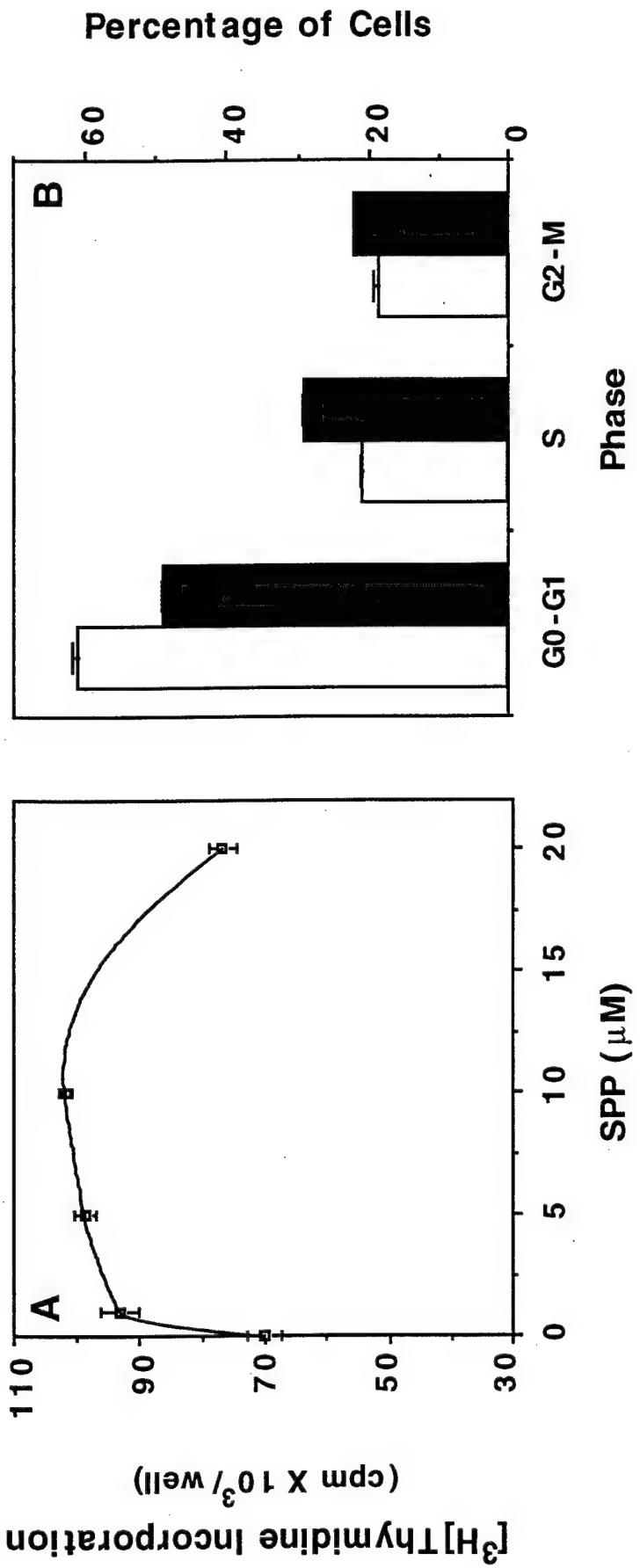
Control
SPP (20 μM)
Sph (20 μM)

Figure 2


Percentage of Cells
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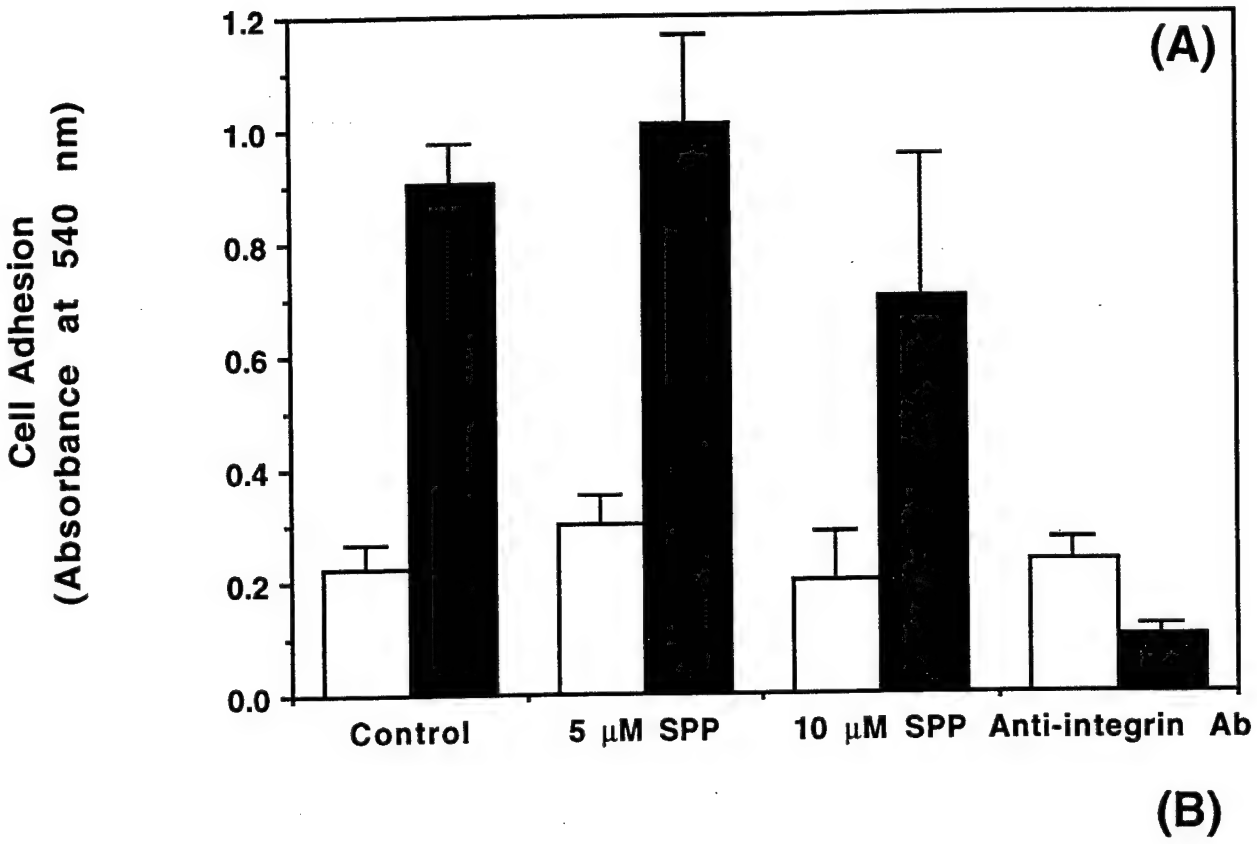
40
20
0
G0-G1
S
G2-M
Phase
A
110
90
70
50
30
0
5
10
15
20
SPP (μM)
[3H]Thymidine Incorporation
(cpm X 10³/ well)

Figure 3


Cell Adhesion
(Absorbance at 540 nm)
1.2
1.0
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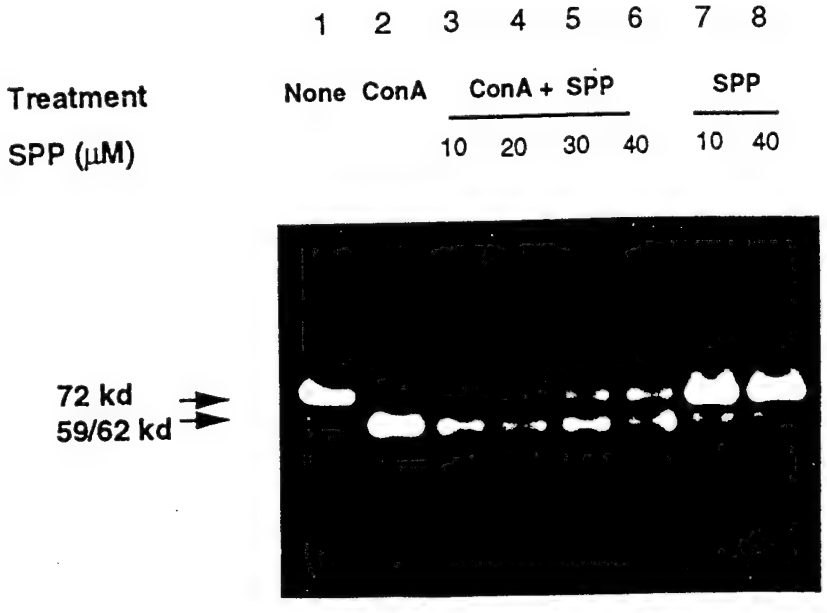
0.6
0.4
0.2
0.0
(A)
Control
5 μM SPP
10 μM SPP
Anti-integrin Ab

(B)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Treatment
None ConA ConA + SPP SPP
SPP (μM)
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72 kd
59/62 kd

Figure 4.

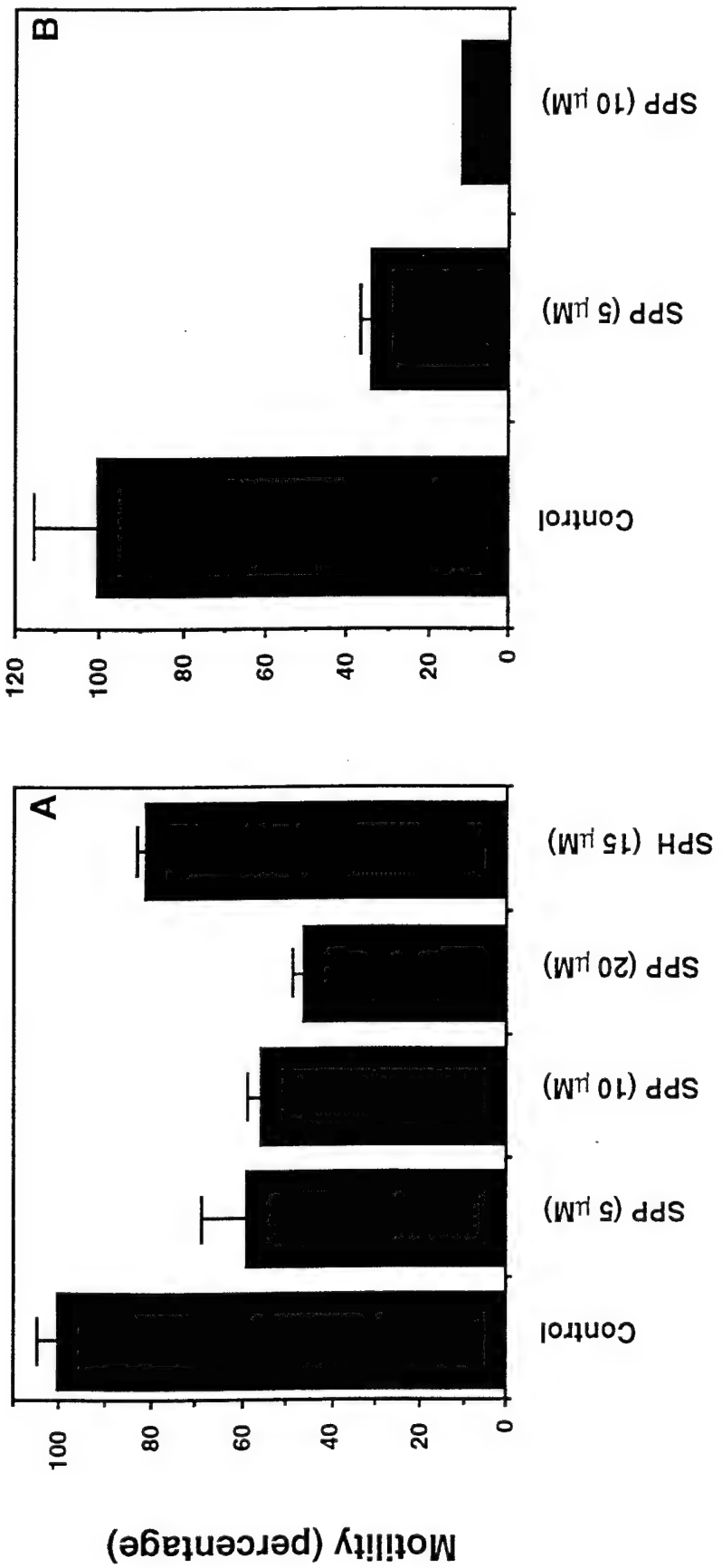
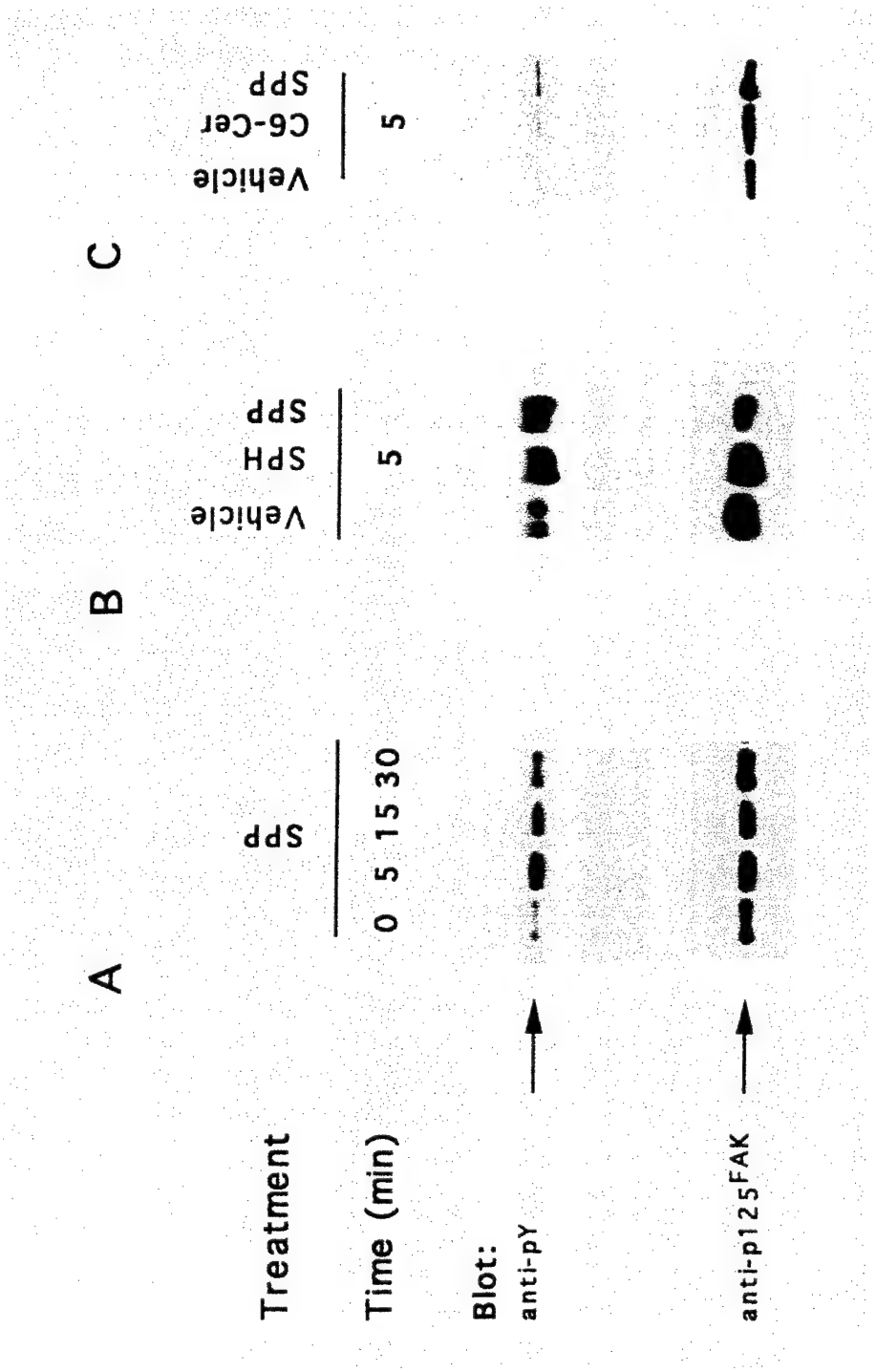

Motility (percentage)
A
0
20
40
60
80
100
Control
SPP (5 μM)
SPP (10 μM)
SPP (20 μM)
SPH (15 μM)
B
0
20
40
60
80
100
120
Control
SPP (5 μM)
SPP (10 μM)

Figure 5.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Treatment
Time (min)
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0 5 15 30
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Vehicle
SPH
Spp
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C
Vehicle
C6-Cer
Spp
5

A
Treatment
Time (min)
SPP
0 5 15 30 60
Blot:
anti-pY
anti-paxillin
B
Vehicle
SPH
SPP
5
C
Vehicle
SPH
SPP
Vehicle
SPH
SPP
15
5
anti-pY
anti-Crk

Figure 6.

Figure 7.

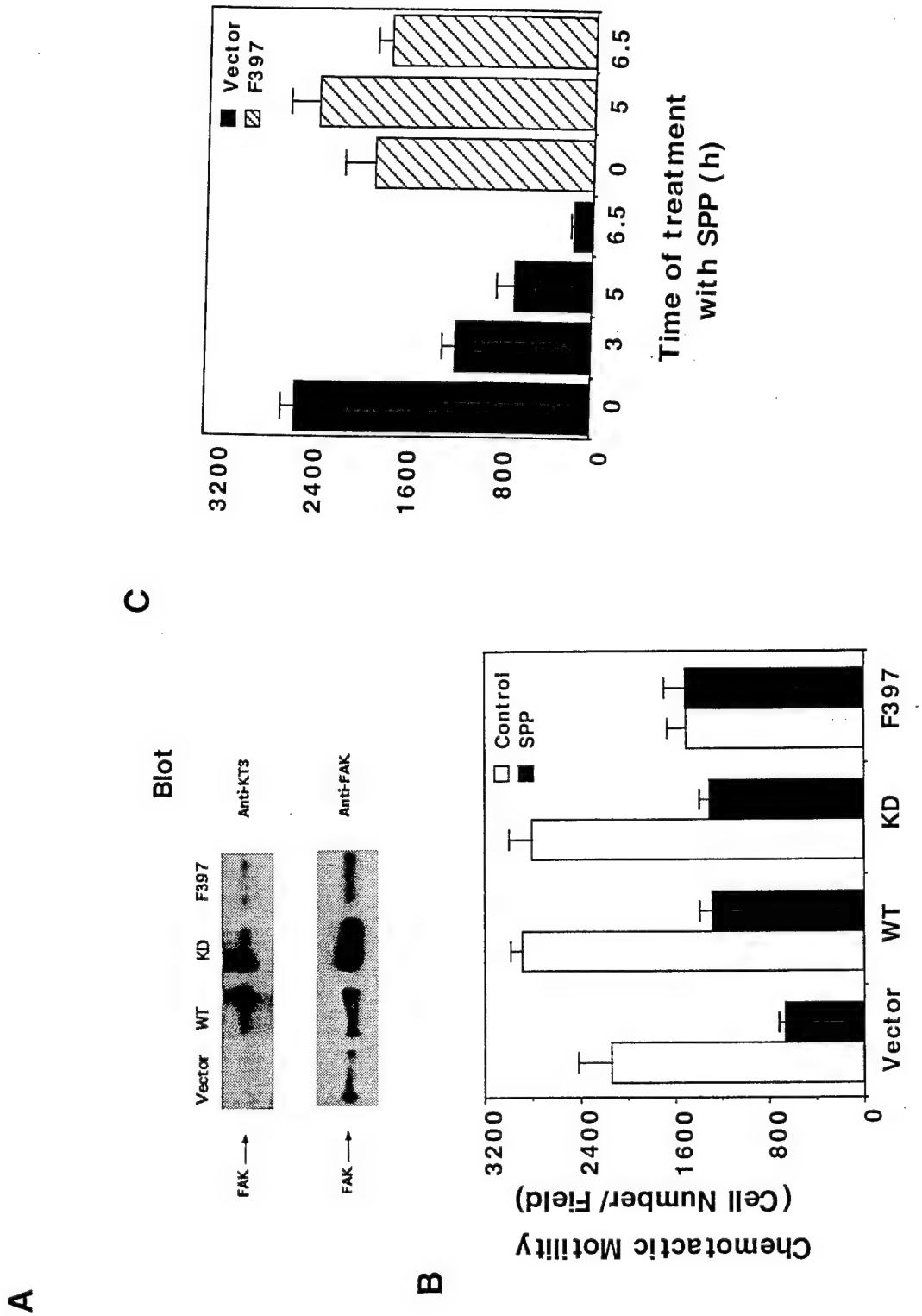
A
Blot
Anti-KT3
Anti-FAK
Vector
WT
KD
F397
FAK
FAK
B
Chemotactic Motility
(Cell Number/ Field)
Control
SPP
3200
2400
1600
800
0
Vector
WT
KD
F397
C
Vector
F397
3200
2400
1600
800
0
0
3
5
6.5
0
5
6.5
Time of treatment
with SPP (h)

FIGURE LEGENDS

Figure 1. SPP reduces chemoinvasion of MDA-MB-231 cells. MDA-MB-231 cells were treated with vehicle (Control), 5 μM N-acetylsphingosine (Cer-2), 5 μM N-hexanoylsphingosine (Cer-6), 1 U/ml sphingomyelinase (SMase) from *Streptomyces sp.*, or the indicated concentrations of SPP and Sph for 24 h, harvested with trypsin, counted and allowed to migrate through polycarbonate filters coated with 25 μg of Matrigel for 6 h in the presence of the indicated agents. Fibroblast conditioned medium (filled bars) or 10% of FBS (open bars) were added to the lower chamber as chemoattractants and chemoinvasion was measured as described in Materials and Methods. Chemoinvasion of control cells was 72 cells/field. The basal chemoinvasion in the absence of chemoattractants was 3 cells/field. The results are from a representative experiment repeated four times. **B.** MDA-MB-231 cells were treated with vehicle (Control), or the indicated concentrations of SPP and Sph during migration for 6 h through polycarbonate filters coated with 25 μg of Matrigel. Fibroblast conditioned medium (filled bars) or 10% of FBS (open bars) were added to the lower chamber as chemoattractants.

Figure 2. Effect of SPP on growth of MDA-MB-231 cells. A. [^{3}H]Thymidine incorporation. MDA-MB-231 cells (1.8 x 10^4 cells/cm^2) were incubated in chemically-defined medium in the absence or presence of various concentrations of SPP for 24 h and [^{3}H]thymidine incorporation was measured as described in Materials and Methods. Similar results were obtained in at least five additional experiments. **B. Flow cytometric analysis of cell cycle distribution.** Cells were incubated in chemically-defined medium in the absence (open bars) or presence of 10 μM SPP (filled bars). After 24 h, cells were analyzed by FACS as described in Materials and Methods. Results (mean ± SD; n=3) are expressed as percentage of cells in each of the cell cycle phases.

Figure 3. Effects of SPP on cell adhesion to Matrigel (A) and ConA-induced activation of metalloproteinase 2 (B). A. MDA-MB-231 cells were treated with vehicle, the indicated concentrations of SPP for 24 h, anti-integrin β1 (20 μg/ml) or unrelated antibody for 1 h, and attachment of cells to plastic (open bars) and to plates coated with Matrigel (filled bars) was determined as described in Materials and Methods. **B.** MDA-MB-231 cells (5 x 10^4 cells/cm^2) were treated with ConA in the absence or presence of SPP (40 μM) in medium containing latent MMP-2. After 24 h, MMP-2 in the

medium was analyzed by zymography as described in Materials and Methods. Latent (72 kD) and mature (62/59 kD) forms of MMP-2 are indicated.

Figure 4. SPP markedly inhibits chemotaxis of MDA-MB-231 and MCF-7 cells. MDA-MB-231 (A) or MCF-7 (B) cells were pretreated with the indicated concentrations of SPP or sphingosine (Sph) for 24 h, harvested, counted and allowed to migrate for 6 h through polycarbonate filters coated with 5 μg of collagen IV. Chemotaxis was measured as described in Materials and Methods. Motility of control cells is 90 and 26 cells/field in MDA-MB-231 and MCF-7 cells, respectively. The basal chemotactic motility in the absence of chemoattractants is 6 and 2 cells/field in MDA-MB-231 and MCF-7 cells, respectively. **C.** MDA-MB-231 cells were treated with vehicle (Control), or the indicated concentrations of SPP and Sph during chemotaxis for 6 h..

Figure 5. SPP induces FAK tyrosine phosphorylation. In panel A, MDA-MB-231 cells were cultured in FBS/IMEM, washed, and treated in chemically-defined medium with 10 μM SPP for the indicated times. In panels B and C, cells were treated for 5 min with the sphingolipid metabolites: SPP (10 μM); Sph (20 μM); C6-Cer (5 μM). Cell lysates were immunoprecipitated with anti-p125^{FAK} mAb and analyzed by immunoblotting with anti-phosphotyrosine (Top) or anti-p125^{FAK} antibody (Bottom). The data are representative results from at least three independent experiments.

Figure 6. SPP stimulates tyrosine phosphorylation of paxillin and Crk. (A) MDA-MB-231 cells were treated with SPP (10 μM) for the indicated times or **(B)** with Sph (20 μM) or SPP (10 μM) for 5 min. Cell lysates were immunoprecipitated with anti-paxillin mAb and the precipitates resolved on SDS-PAGE gels, transblotted and analyzed by Western blotting with anti-Tyr(P) antibody (top) or anti-paxillin antibody (bottom). **(C)** MDA-MB-231 cells were treated with SPP (10 μM) or Sph (20 μM) for 5 min. Lysates were immunoprecipitated with anti-Crk mAb and analyzed by Western blotting with anti-Tyr(P) antibody (top) or anti-Crk antibody (bottom).

Figure 7. SPP does not inhibit chemotaxis of MDA-MB-231 cells expressing autophosphorylation site mutated FAK. A. Cells overexpressing vector alone or wild type (WT), kinase defective (KD) or autophosphorylation site mutated (F397) FAK were lysed and analyzed by Western blotting with anti-KT3 (upper panel) or anti-FAK mAb (lower panel). **B.** Chemotaxis of cells overexpressing wild type or mutated FAK was determined in the presence of SPP (15 μM) (closed bars) or vehicle (open bars) added to the lower chamber containing fibroblast conditioned medium. Cells were allowed to

migrate for 4 h through polycarbonate filters coated with 5 μg of collagen IV and chemotaxis was measured as described in Materials and Methods. **C.** Chemotaxis of cells expressing vector (open bars) or F397 FAK (streched bars) was determined after 6 h migration as in B. Cells were either treated with SPP (15 μM) for 3 h, 2 h prior to the start (6.5 h, 5 h) or was added during the assay (3 h). For comparison, migration of cells was determined in the absence of SPP (0 h).

DISCUSSION

SPP, inhibits chemoinvasiveness of the aggressive, estrogen-independent MDA-MB-231 HBC cell line. Three critical steps have been identified in the process of invasion of cancer cells: attachment of tumor cells to the basement membrane, extracellular matrix digestion, and subsequent cellular movements [25]. SPP had no significant effect on the adhesiveness of cells to Matrigel, an extract rich in basement membrane components. Similarly, others have found that although SPP inhibits integrin-dependent motility of mouse melanoma B16 cells, it does not reduce integrin-dependent adhesion to the extracellular matrix [19]. Penetration of the extracellular matrix by metastatic cells requires disruption of local segments of the basement membrane by proteinases, such as matrix metalloproteinase 2 (MMP-2) which has been proposed to play an important role in invasion due to its specificity for basement membrane collagen [28]. We found that only high concentrations of SPP inhibited ConA-induced MMP-2 activation by MDA-MB-231 cells. These results suggest that neither attachment nor MMP-2 activation are critical targets for SPP-dependent inhibition of chemoinvasion. In contrast, one of the critical steps for the invasion of cancer cells, cell motility, was markedly inhibited by SPP. In B16 melanoma cells, SPP strongly inhibited cell motility and phagokinesis at low concentrations (10-100 nM), whereas sphingosine or other related sphingolipids were inactive [19]. Similarly, only SPP inhibited integrin-dependent motility of melanoma cells induced by ECM, suggesting that SPP might be acting extracellulary via a cell surface receptor [17, 35]. In contrast, we found that sphingosine, which is readily taken up by cells and phosphorylated to SPP [21], also inhibited motility and invasion of MDA-MB-231 cells, but to a lesser extent than SPP. Moreover, the inhibitory effects of SPP were observed only at concentrations of 5-20 μM. Thus, SPP may act intracellularly, and not by binding to a putative cell surface receptor, to inhibit motility and invasion of MDA-MB-231 cells. This is consistent with the

conclusion that SPP, formed in response to PDGF, plays an important role in PDGF-regulated motility of human arterial smooth muscle cells [16].

It was reported that an increased level of FAK expression is highly correlated with invasion potential of human tumor cells [36, 37], suggesting that FAK may be limiting for cell invasion. FAK has been indicated to play a crucial role in cell migration events [10, 31, 38, 39]. Cultured fibroblasts isolated from FAK-deficient mice, display reduced cell motility and enhanced focal adhesion contact formation [40]. In agreement, displacement of endogenous FAK from focal adhesions by microinjection of its carboxy-terminal domain in endothelial cells resulted in decreased cell migration [38]. Overexpression of FAK, or a kinase-defective FAK mutant, promoted migration of CHO cells on fibronectin [10]. In agreement, we found that overexpression of wild type or kinase defective FAK slightly increased chemotactic motility of MDA-MB-231 cells.

Previously, in Swiss 3T3 fibroblasts, it was shown that SPP, and sphingosine also stimulated tyrosine phosphorylation of FAK, paxillin, and Crk [33], leading to stress fiber and focal contact formation [7, 15]. Yet the relevance of these observations to motility of Swiss 3T3 fibroblasts has not been determined. However, ECM-dependent FAK activity was not altered in SPP-inhibited haptotactic motility of mouse B16 melanoma cells [35]. We found that SPP and sphingosine, but not ceramide, increased tyrosine phosphorylation of FAK in MDA-MB-231 cells with a concomitant increase in tyrosine phosphorylation of paxillin and Crk. This observation is intriguing since it has been shown before that increased tyrosine phosphorylation of FAK correlates with increased cell motility in EGF [39] or PDGF [41] induced cell movement. However, it was shown that NIH 3T3 cell lines overexpressing *v-Src* exhibited a dramatically reduced migration rate and increased FAK phosphorylation compared with control NIH 3T3 cells. In the case of NIH 3T3 cell line overexpressing the kinase mutant form of *v-src*, although the extent of FAK tyrosine phosphorylation was lower than control NIH 3T3 cells, this cell line exhibited a modest reduction in the migration rate compared with control cells [31]. Thus there appears to be an optimal level of FAK tyrosine phosphorylation required for maximal migration.

Previously, Guan and co-workers demonstrated that overexpression of FAK, or a kinase-defective FAK mutant that was tyrosine phosphorylated and associated with Src, promoted migration of CHO cells on fibronectin [10]. This effect was dependent on FAK autophosphorylation at Y397 and subsequent binding of Src to this site. We found

that overexpression of wild type and kinase-defective FAK in MDA-MB-231 slightly increased chemotactic motility, without influencing the inhibitory effect of SPP. In contrast, expression of autophosphorylation site mutated FAK abolished the effect of SPP on motility. This suggests that the autophosphorylation site on FAK may play an important role in SPP mediated signaling leading to decreased cell motility.

Autophosphorylation of FAK at Y397 leads to its association with Src, resulting in activation of both kinases (review see [14]). The activated FAK/Src complex phosphorylates several substrates, including tensin, paxillin and p130cas.

Inhibition of cell spreading by expression of the C-terminal domain of FAK is rescued by coexpression of Src or catalytically inactive FAK with concomitant enhancement of tyrosine phosphorylation of paxillin [42]. These results suggest that tyrosine phosphorylation of paxillin is a critical step in focal adhesion assembly. In this scenario, FAK may act as a "switchable adaptor" that recruits Src to phosphorylate paxillin, thus promoting focal adhesion assembly.

FAK phosphorylation and/or binding to paxillin and p130cas may trigger downstream activation of MAP kinase by the adoptor protein Crk. Src association with FAK may also result in further FAK phosphorylation, forming a docking site for Grb2. However recent results [11] demonstrate that p130Cas, but not Grb2, is a mediator of FAK-promoted CHO cell migration. FAK-induced p130Cas phosphorylation occurred when a kinase-defective but not Y397F FAK mutant was expressed indicating that Src may be the kinase that mediates phosphorylation of p130Cas [43]. This result also suggests that the inability of the Y397F FAK mutant to promote cell migration may be due to inefficient p130Cas phosphorylation and recruitment of signaling molecules. Moreover, it was demonstrated that the catalytic activity of Src is dispensable for translocation to focal adhesions but controls the turnover of these structures during cell motility [44]. Thus, it was proposed that the Src-FAK-linked activity induces focal adhesion turnover and consequently, loosens cellular adhesion strength, and facilitating cell movement.

Further studies are needed to clarify the importance of Src in the mobility inhibiting effect of SPP. However, since Y397 can also bind PI 3-kinase, which might be important for motility [45, 46] , the possibility cannot be excluded that PI 3-kinase is also important for SPP signaling.

Although we found that the autophosphorylation site on FAK was important in SPP-inhibited chemotactic motility of MDA-MB-231 cells, other signaling pathways may play important roles in other cell lines. In human smooth muscle cells, SPP induced actin filament disassembly and inhibited chemotaxis toward PDGF. While tyrosine phosphorylation of FAK was not affected, SPP induced increase in cyclic-AMP levels and activation of cAMP-dependent protein kinase. Part of the effect of SPP on migration was mediated through activation of PKA [16].

CONCLUSIONS

1. Sphingosine 1-phosphate inhibited invasion and motility of human breast cancer cell MDA-MB-231.
2. SPP rapidly increased tyrosine phosphorylation of FAK and paxillin and of the paxillin-associated protein Crk.
3. The autophosphorylation of FAK on Y397 may play an important role in SPP signaling leading to decreased cell motility.

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